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(54) Title: SELECTIVE CELL SEPARATION (57) Abstract A method for separating a mixture of cells in suspension is described. A suspension of cells is provided which has a first cell type and a second cell type. A porous material which has a bound ligand is also provided. The bound ligand is able to additionally bind to cells of the first cell type. The suspension of cells is contacted with the porous material which has the bound ligand under conditions which allow cells of the first cell type to bind to the bound ligand so as to form a porous material-ligand-cell complex. This complex is treated so as to substantially remove the second cell type from the porous material. A back pressure is applied across the porous material-ligand-cell complex so as to detach the first cell type from the complex, and the detached cells are then recovered.		

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SELECTIVE CELL SEPARATION

Field of the Invention

This invention relates to methods for selectively separating specific cell types from a
5 mixture of cells in suspension, and more particularly to methods for detaching cells adsorbed to porous materials.

Background of the Invention

The selective separation of a specific cell population from a mixture of cells is important
10 in a variety of situations, including certain medical therapies and biotechnological applications.

Current separation methods include affinity separation techniques which make use of the fact that different cell types are characterized by different cell surface markers and that specific ligands bind to specific cell surface markers. Affinity separation techniques have been employed in various ways, including: (i) adding ligands in the form of soluble polymers to a cell
15 suspension to bind to the markers on the cells and precipitating the cells; (ii) immobilizing the ligand to a solid support (e.g., beads, magnetic beads, petri dishes, cell culture flasks, a packed bed of beads, fibers, films, and other porous or nonporous materials), which is then used to remove cells having the appropriate markers from a cell suspension; and (iii) attaching the ligand to the walls of a conduit which is composed of a solid film or a semi-permeable membrane,
20 through which the cell suspension is made to flow.

These techniques suffer from various shortcomings, in that some are labor intensive, time consuming, have low selectivity due to non-specific entrapment of undesired cells, have a low yield of desired cells, or are not practical for clinical scale cell loads. In addition, standard means which have been used in the various affinity separation methods to detach the cells from the
25 ligands to which they have been bound (e.g., chemical elution or application of a large uncontrolled physical force), often result in damage to, or death of, the cells.

Summary of the Invention

It is an object of the invention to provide an improved technique for the selective
30 separation of a specific cell population from a mixture of cells.

It is another object of the invention to provide a method for the separation of a specific cell population from a mixture of cells which gives high selectivity for the desired specific cell

population.

It is yet another object of the invention to provide a method for the selective separation of a specific cell population from a mixture of cells such that a high proportion of the recovered cells are viable.

5 Still another object of the invention is to use back pressure to effect detachment of cells that are bound to a porous material.

The invention includes a method for separating a mixture of cells in suspension having at least a first cell type and a second cell type. A porous material, e.g., a membrane, is provided, which has a bound ligand, the bound ligand being able to additionally bind to cells of the first
10 cell type. The suspension of cells is contacted with the porous material which has the bound ligand under conditions which allow cells of the first cell type to bind to the bound ligand so as to form a porous material-ligand-cell complex. This complex is treated, e.g., washed, so as to substantially remove the second cell type from the porous material. A back pressure is applied across the porous material-ligand-cell complex so as to detach the first cell type from the
15 complex. The detached cells are then recovered. Preferably, a substantial portion of the detached cells are viable.

In certain embodiments, an additional parameter, e.g., a shear force, an electrical force, a centrifugal force, a chemical, a biological molecule, a pH change, and/or an ionic strength change, is applied to aid in detachment of the first cell type.

20 Another aspect of the invention features a method for detaching cells that are adsorbed to a porous material. A porous material having adsorbed cells is provided. A back pressure is applied across the porous material so as to detach the adsorbed cells from the porous material, and the detached cells are recovered. It is preferred that the porous material has a bound ligand and that the cells are adsorbed to the porous material as a result of the cells being bound to the
25 ligand.

In certain embodiments, the adsorbed cells comprise a first cell type and a second cell type, with the first cell type being adsorbed to the porous material with a higher affinity than the second cell type. A second back pressure across the porous material is applied after application of a first back pressure such that the first back pressure selectively detaches the second cell type
30 and the second back pressure selectively detaches the first cell type.

The above and other objects, features and advantages of the present invention will be better understood from the following specification when read in conjunction with the

accompanying drawings. It is to be understood that the drawings are designed for the purpose of illustration only and are not intended as a definition of the limits of the invention.

Brief Description of the Drawings

5 FIG. 1 is a diagrammatic view of a functionalized hollow fiber membrane employed in the Examples;

FIG. 2 is a diagrammatic view of a pressurization assembly using a petri dish; and

FIG. 3 is a diagrammatic view of a pressurization assembly using a centrifuge tube.

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Detailed Description

This invention provides a method for separating a mixture of cells in suspension having at least a first cell type and a second cell type. A porous material is provided having a bound ligand, the bound ligand being able to additionally bind to cells of the first cell type. The suspension of cells is contacted with the porous material which has the bound ligand under
15 conditions which allow cells of the first cell type to bind to the bound ligand so as to form a porous material-ligand-cell complex. This complex is treated so as to substantially remove the second cell type from the porous material. A back pressure is applied across the porous material-ligand-cell complex so as to detach the first cell type from the complex. The detached cells are then recovered.

20 Cells are meant to include animal cells, plant cells and microorganisms. By cell type is meant a cell which is capable of being distinguished from other cell types based on its binding affinity for a given ligand under given conditions. For example, a first cell type could be an animal cell and a second cell type could be a plant cell, or a first cell type could be a cell from an animal and a second cell type could be a cell from a different type of animal, or a first cell type
25 could be from one tissue and a second cell type could be from a different tissue or a different part of the same tissue or the same part of the same tissue in the same animal, or a first cell type could be a stem cell and a second cell type could be a fully developed cell from the same animal, or a first cell type could be a normal cell and a second cell type could be a cancerous form of the normal cell or some mutated form of the normal cell. The invention is meant to cover any
30 mixture of different cell types. The mixture can have two or more different cell types. The cells can be suspended in any liquid, e.g., a naturally occurring liquid, e.g., blood, lymph, bone marrow aspirate, saliva or urine, or they can be suspended in a solution, e.g., a buffer. Other

materials may also be present in the suspension.

By porous material is meant any material which has openings through which a pressurized gas or liquid can be administered to detach target cells from the porous material.

Examples of a porous material are a membrane or a filter paper. The porous material includes, 5 e.g., any polymeric material which is able to bind a ligand or is capable of being modified so as to be able to bind a ligand, or which is able to bind a cell or is capable of being modified so as to be able to bind a cell. Examples of organic porous materials include polysulfone, polyethersulfone, polyacrylonitrile, polyamide, e.g., nylon, polycarbonate, polyacrylate, polyvinylidene fluoride, polyethylene, polypropylene, polyimide, poly(arylene oxide), 10 poly(arylene sulfide), polyquinoxaline, polyurethane, poly(etheretherketone), polyester, poly(vinyl halide), poly(vinylidene polyhalide), cellulose, e.g., cellulose, regenerated cellulose, cellulose acetate, cellulose nitrate, mixed esters of cellulose, and polytetrafluoroethylene (Teflon®). Examples of inorganic porous materials include glass fiber filter paper, ceramic membranes, e.g., alumina or silica, metal filters, sintered glass and sintered ceramic blocks. 15 Derivatives, blends, mixtures and copolymers of the porous materials can also be used. The porous material can be, e.g., anisotropic or isotropic. Anisotropic materials having smaller pores and/or porosity in the surface region than in the bulk material are preferred so as to minimize flow within the plane of the surface of the porous material to which the target cells are bound. It may be preferable that the porous material be biocompatible.

20 Preferred porous materials are membranes, e.g., semi-permeable membranes. Membranes are meant to include, e.g., microfiltration membranes and ultrafiltration membranes. The membranes can be in various configurations, e.g., hollow fibers, tubes, or flat sheets. The membrane can be, e.g., formed into, or be part of, a conduit through which the cell suspension can flow. A variation includes the membrane being placed on a moving surface, such as the 25 rotating inner cylindrical core of an arrangement analogous to a Couette viscometer, as well as on the outer cylinder of the same arrangement. Another variation includes the membrane being placed on the stationary or moving surface of a configuration of a rotating disc in a housing. In certain embodiments, a single membrane is used and in other embodiments multiple membranes contained within a single module are used. Examples of such modules include multiple hollow 30 fibers in a shell and tube arrangement, or multiple flat sheets stacked in a cassette or wound in spirals. Configurations having multiple membranes are preferred for applications in which large amounts of cells need to be separated.

Preferably, the porous material has pores of a size so as to preclude passage of the cells through the surface of the porous material and to preclude lysis of the cells resulting from substantial deformation of the cell membrane through the surface pores. The pore diameter of the surface to which the target cells are bound is preferably in the range of about 0.005 microns to about 3 microns, and most preferably is in the range of about 0.01 microns to about 1 micron.

A ligand is bound to the porous material. By ligand is meant any molecule which is able to bind to the porous material directly, or to a modified porous material, and which is additionally able to selectively bind to a particular cell type. Examples of ligands include monoclonal and polyclonal antibodies, integrins, selectins, cytokines, lectins such as concanavalin A, antigens or haptens to cell surface antibodies, hormones, fibrin, collagen, laminin, fibronectin, vitronectin, adhesion peptides, avidin, biotin, streptavidin, protein A and protein G. Preferably, the ligand is covalently bound to the surface of the porous material.

Binding of the ligand to the porous material is meant to include binding directly to the porous material itself, or binding to a modified porous material. A modified porous material is meant to include, e.g., a porous material which has been coated on the surface with some material, e.g., a hydrophilic polymer. This coating can then be functionalized with chemically reactive groups so as to facilitate binding to a ligand. For example, the coating can be functionalized so as to result in a surface containing hydrazide groups which are reactive with aldehyde groups, or the coating can be functionalized so as to result in a surface containing 2-alkyloxy-1-methylpyridinium salts which are reactive with nucleophiles such as amino or sulfhydryl groups. Other coatings and functionalizations can be used as are known to those skilled in the art, which result in the generation of chemically reactive groups on a given porous material such that they can react with and bind to a given ligand of interest. See, e.g., Hermanson, G.T., Mallia, A.K. and Smith, P.K., "Immobilized Affinity Ligand Techniques", Academic Press, Inc., San Diego, CA (1992); Klein, E., "Affinity Membranes: Their Chemistry and Performance in Adsorptive Separation Processes", J. Wiley and Sons, New York (1991). Certain types of pre-functionalized porous membranes are commercially available (e.g., from Arbor Technology, Ann Arbor, MI; Millipore, New Bedford, MA). In some embodiments, the ligand can be directly bound to a porous polymer containing reactive surface groups, e.g., amines or hydroxyl groups, without the need to add an additional functionality or surface coating.

The ligand can be bound to the internal surface of the porous material so long as it is bound to at least one external surface of the porous material. By internal surface is meant the

surface area within the porous membrane itself, i.e., the surface of the solid structure which defines the internal pores or interstices. Preferably, it is bound to one external surface of the porous material. For example, if the porous material is a flat sheet membrane, the ligand is preferably bound to one external surface of the sheet; if the porous material is a hollow fiber membrane, the ligand is preferably bound to the surface of the lumen or to the outer surface of the fiber.

The density of ligand that is bound to the porous material is preferably about 10^7 molecules/cm² to about 10^{13} molecules/cm², and most preferably is about 10^9 molecules/cm² to about 10^{12} molecules/cm².

10 Binding of the bound ligand to cells is generally mediated by cell markers on the surface of the cells, such as antigenic epitopes or cell surface receptors, but any other naturally occurring or modified molecular structure or condition of the cell that involves one or more molecules or portions thereof which permits selective binding of the given ligand to the given cell type can be utilized. Different cell types are characterized by different cell surface markers. By employing a
15 ligand which binds to a specific marker, cells containing that marker are selectively separated from a suspension of different cell types. The ligand can be directed, e.g., to a broad class of markers present on many cells, e.g., lectin binds to hormone receptors that are present on a broad class of cells. An alternative may be that the ligand is directed to a more specific class of markers, e.g., a monoclonal antibody binds to a specific antigenic epitope that may be present on
20 only a specific cell type.

The invention is also meant to include indirect binding of the cell to the ligand via an intermediary molecule ("sandwich" methods). For example, the cells are preincubated with epitope-specific monoclonal antibodies so that these antibodies are bound to all or a fraction of the cell marker sites. The cells are then selectively bound to the porous material having a bound
25 ligand which reacts with the monoclonal antibody (as opposed to the epitope directly), e.g., anti-mAb antibodies or another binding ligand.

Contacting of the porous material having the bound ligand with the suspension of cells can be accomplished by any procedure which results in binding of the first cell type to the bound ligand. For example, the cell suspension can be introduced by flow onto the surface of the
30 porous material, or the porous material can be immersed in the cell suspension. The cells can settle onto the surface of the porous material by gravity or they can be convected to the surface by applying, e.g., forward pressure across the porous material so as to induce normal filtration.

If a cell colliding with the surface is positioned so that a surface-bound ligand on the porous material is in close proximity to a targeted cell surface marker for a sufficient length of time, a bond will form between the ligand and the marker site. If all of the bonds which form are strong enough to resist the action of any shear forces that are present, generally the cell will remain adherent to the ligand-coated surface. Cells which contain no surface markers that are specific for the ligand will not bind to the ligand-coated surface and, if in contact with that surface, will in general be displaced by shear forces and/or collisions with the other cells. It may be preferable to carry out the contacting step without flow of the cell suspension and with only a forward pressure drop across the porous material. The loading of cells onto the surface of the porous material can result in a layer of cells which covers substantially the entire surface or which only partially covers the surface. It may be preferable for the concentration of cells to be large enough so that more than a monolayer of cells is created on the surface of the porous material. In such a situation more bonds can form between the cell surface markers and the ligand-coated surface because the bed of cells which builds up exerts pressure on the underlying surface layer of cells which results in deformation of the cells so as to put more cell surface in contact with the porous material.

It is desirable to increase the strength of attachment of specifically-bound cells so that they are not easily detached by moderate shear forces and are held more strongly than any cells which become attached to the surface by non-specific intermolecular forces. The strength of attachment of cells that bind specifically is proportional to the number of bonds which form between the cell markers and the ligands on the membrane surface. It may be preferable that the cells be incubated under static conditions for enough time to allow for possible receptor diffusion into the contact area and formation of a sufficient number of bonds to hold the cells on the porous material during the subsequent washing step. Preferably, such incubation is about 0.1 minutes to about 60 minutes, and most preferably is about 0.2 minutes to about 15 minutes. Binding may also be enhanced by imposing forward pressure across the porous material during incubation so that the cells may be flattened against the porous material, thereby creating a larger contact area for bonding. In certain embodiments where the cell type of interest constitutes only a small fraction of all of the cells that are present in the cell suspension, the steps of contacting the cells with the porous material and increasing the strength of attachment can be alternated in rapid sequence, with intermittent dislodging of non-specifically bound cells, so as to attain a higher surface coverage of specifically bound cells before proceeding with the subsequent steps.

The porous material-ligand-cell complex that results is treated so that substantially only specifically bound cells, i.e., cells held by the marker-ligand bonds, remain on the porous material surface. Such treatment includes, e.g., washing the complex, though any treatment which substantially removes non-targeted cells which become attached to the surface by weaker non-specific forces, as well as substantially removing all cell types from the bulk suspension not at the surface, can be used. By substantially removing non-targeted cells is meant that at least about 70% of such cells are removed, preferably at least about 80%, more preferably at least about 90%, more preferably yet at least about 95%, and most preferably at least about 99%. Preferably, the complex is washed by flowing a cell-free liquid which is not harmful to the cells across the surface of the porous material. If forward pressure across the porous material is used in the previous step, such pressure is discontinued. In certain embodiments, a small amount of back pressure is imposed across the porous material to induce backfiltration so that the non-specifically bound cells are subjected to, e.g., hydrostatic and hydrodynamic forces which can aid in removing them from the surface. The amount of back pressure is chosen such that the more strongly bound targeted cells are not removed from the surface in this treatment step.

In order to detach the specifically bound cells from the surface, a back pressure across the porous material-ligand-cell complex of sufficient magnitude to rupture the specific bonds which had formed during the incubation and washing periods is imposed. By back pressure is meant imposition of a pressure drop across the membrane such that the pressure is lower on the surface to which the target cells are bound and higher on the opposing surface, thereby inducing back filtration. In contrast, by forward pressure is meant imposition of a higher pressure on the side of the membrane to which the cells become bound, thereby inducing normal filtration. The back pressure can be imposed using, e.g., a liquid or a gas, preferably a liquid. Preferably, the pressure imposed is about 0.2 psig to about 100 psig, and most preferably is about 1 psig to about 30 psig. The detachment force resulting from the imposition of back pressure which is exerted on a cell bound to the surface of a porous material is a function of several variables, such as the size of the contact area between the cell and the porous surface, the distance between the cell contact area and the porous surface, and the hydraulic permeability of the porous material. Preferably, the porous material has a sufficiently high hydraulic permeability such that the preferred back pressure can create sufficient hydrostatic and hydrodynamic forces on the bound cells in order to rupture the specific bonds and remove the cell from the porous surface. The detached cells are then recovered. Preferably, the cells are detached in a liquid environment so

that the cells become suspended in the liquid. The liquids that can be used include, e.g., phosphate buffered saline, culture media, or balanced salt solutions. An advantage of the detachment procedure used in this invention is that a substantial portion of the detached cells are viable. Preferably, of the cells that were viable in the original suspension before separation, 5 greater than about 50% of these cells remain viable, more preferably, greater than about 75%, more preferably yet, greater than about 90%, and most preferably, greater than about 95%.

In certain embodiments, one or more additional parameters are applied to aid in the detachment of the specifically bound cells. These parameters include, e.g., a shear force, an electrical force, a centrifugal force, a chemical, a biological molecule, e.g., a proteolytic enzyme 10 or a competing antibody, and a change in ionic strength or pH. In some cases, a change in the solution environment of the cells is preferably carried out by incorporating the change in the liquid used for back filtration. For example, a low pH solution can be used as the back filtration solution while a physiological buffer is flowed over the surface containing the cells such that the cells are exposed to low pH for a very short period of time.

15 In certain embodiments, the above-described method is repeated multiple times so as to reduce the porous material surface area that is required for the separation of a given amount of cells.

In certain embodiments, pressures and/or flow rates of the fluids on each side of the membrane are automatically controlled by an electronic or mechanical process control system.

20 It is advantageous to use this invention for any application which requires high selectivity for the separation of specific cell types from mixed cell populations and/or which requires that a high percentage of the recovered cells be viable. The selective separation methods of this invention have innumerable uses in medical therapies and biotechnology applications. For example, they are useful in the removal of cancer cells or T lymphocytes from bone marrow 25 grafts; in the selection of stem cells from autologous bone marrow for engrafting in cancer patients undergoing bone marrow transplanation; in the selection of specific subpopulations of white blood cells for immunological uses; in the selection of red blood cells for transfusions; in the selection of antigen-specific hybridomas for monoclonal antibody production; in the selection of pancreatic islet cells for transplantation; in the removal of antigen-presenting cells from tissue 30 grafts to prevent or delay rejection; in the removal of HIV-infected cells for AIDS treatment; and in the isolation of stem cells from bone marrow or peripheral blood in the treatment of malignancies and leukemias.

The invention also includes a method for detaching cells that are adsorbed to a porous material. A porous material having adsorbed cells is provided. A back pressure is applied across the porous material so as to detach the adsorbed cells from the porous material, and the detached cells are recovered. Preferably, the porous material has a bound ligand, and the cells are
5 adsorbed to the porous material as a result of the cells being bound to the ligand.

In certain embodiments, two cell types are adsorbed to the porous material, with the first cell type being adsorbed with a higher affinity than the second cell type. A second back pressure across the porous material is applied after application of a first back pressure such that the first back pressure selectively detaches the second cell type and the second back pressure selectively
10 detaches the first cell type. This procedure is also applicable to more than two different cell types which have differential adsorption affinities.

Examples

Example 1: Functionalization of the Membranes

15 Experiments performed utilized polyethersulfone hollow fiber membranes (obtained from Sepracor Inc., Marlborough, MA). Two lots of hollow fibers were employed, designated by the manufacturer as lots XC3089B1 and X3257B2. The hollow fibers were coated with polymers containing hydroxyl groups or imine groups and further functionalized with chemically reactive groups in order to reduce non-specific adsorption and facilitate the immobilization of protein.
20 The membranes were functionalized by one of the following procedures. Some of the procedures followed are described in Azad, A.R.M. and Goff, R.A., Process for the Covalent Surface Modification of Hydrophobic Polymers and Articles Made Therefore, PCT International Publication No. WO 90/04609, published May 3, 1990, and Ngo, T.T., Facile Activation of Sepharose Hydroxyl Groups by 2-Fluoro-1 Methylpyridinium Toluene-4-Sulfonate: Preparation
25 of Affinity and Covalent Chromatographic Matrices, BIO/TECHNOLOGY, 4:134-137 (1986).

(a) Hydrazide-functionalized fibers

Polyethylene imine (PEI) was coated onto the hollow fiber membrane to provide the imine groups following treatment of the membrane with ethylene glycol diglycidyl ether (EGDGE). The membrane was then treated with glutaraldehyde and then adipic acid
30 dihydrazide, which resulted in a surface containing hydrazide groups which were reactive with aldehyde groups.

Functional hydrazide groups on hydrazide-functionalized fibers were assayed by reaction

of the fibers with ninhydrin reagent solution (Sigma, St. Louis, MO) which reacts with primary and secondary amines forming a soluble product which absorbs at 570 nm. Absorbance of the reaction solution was compared to solutions containing known quantities of adipic acid dihydrazide. The average concentration measured with both lots was 12.5 μmol active hydrazide groups per ml of total membrane volume (mlmv). Total membrane volume was calculated as described in Example 2(a).

(b) FMP-functionalized fibers

Hydroxyethylcellulose (HEC) was coated onto the hollow fiber membrane to provide the hydroxyl groups following treatment with EGDGE. The membrane was then treated with triethylamine and 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP) in anhydrous acetonitrile, which resulted in a surface containing 2-alkyloxypyridinium salts which were reactive towards nucleophiles, such as amino groups.

FMP-functionalized fibers were assayed for FMP activity by reaction of the fibers with 6% v/v ethanolamine in water. The soluble product formed from the reaction between the active surface groups and the ethanolamine, 1-methyl-2-pyridone, was detected by its strong absorbance at 298 nm. The extinction coefficient of this compound at 298 nm of 5.9 was used to determine the amount present which is equal to the amount of FMP present on the fiber sample. The concentration measured with lot X3257B2 was 35.5 μmol active FMP groups/mlmv.

20 Example 2: Characterization of the Non-functionalized and Functionalized Membranes

(a) Hollow fiber dimensions and structure

Hollow fibers were sectioned and examined by light microscopy. Average inside diameter was 600 μm and average outside diameter was 1000 μm for both lots. Total membrane volume was defined as:

$$mv = \frac{\pi L}{4} (d_o^2 - d_i^2)$$

25 where d_o is the outer diameter of the fiber, d_i is the inner diameter of the fiber, and L is the length of the fiber sample. Total membrane volume equaled 0.005 mlmv per centimeter of fiber length.

Membrane structure of base non-functionalized fiber was observed with gold-coated sections by scanning electron microscopy (SEM).

All hollow fibers were anisotropic with a surface region on the outside of the fiber which

had substantially lower porosity and pore size than did the bulk of the membrane. Pore size estimation from a small number of surface micrographs gave pore diameters ranging from 0.01 to 0.07 μm for lot XC3089B1 and 0.02 to 0.10 μm for lot X3257B2.

Pore size distribution of the base fibers was also measured using a Coulter® porometer (Coulter Electronics Limited, Luton, England) with Coulter porofil as the wetting fluid. The minimum pore diameter measurable was about 0.07 μm . For XC3089B1 fibers, all pore diameters were smaller than 0.174 μm and the mean flow pore size (defined as the pore size giving the same pressure-flow behavior for a fiber with all pores the same size) was 0.088 μm . The X3257B2 fibers had a pore size range of 0.08 μm to 3 μm with a mean flow pore size of 1.25 μm . Only about 0.5% of the pores were larger than 1 μm , and about 50% of the measured pores were between 0.08 and 0.12 μm .

(b) Surface area

Surface area of the base fibers was measured by gas adsorption/desorption using a Micrometrics Flowsorb II Model 2300 (Micrometrics Instrument Cor., Norcross, GA). The surface area determined in this fashion was used to calculate the surface density of chemical groups and immobilized ligand on the fibers.

The surface area measured for the XC3089B1 fibers was 7.9 $\text{m}^2/\text{ml mv}$, and for the X3257B2 fibers was 4.1 $\text{m}^2/\text{ml mv}$.

(c) Hydraulic flux

Hydraulic flux was measured in wetted fibers by preparing small modules containing the fiber to be measured and pumping a measured volume of water, or phosphate buffered saline, while simultaneously measuring the pressure drop. These measurements were used to determine the hydraulic flux and hydraulic permeability. Hydraulic permeability was measured in both the base non-functionalized fibers and the functionalized fibers. With base fibers, hydraulic permeability was 1.0 $\text{Lm}^{-2}\text{hr}^{-1}\text{mmHg}^{-1}$ for lot XC3089B1 and 3.2 $\text{Lm}^{-2}\text{hr}^{-1}\text{mmHg}^{-1}$ for lot X3257B2. For fibers functionalized with FMP, surface functional groups were first capped with glycine or the fiber was treated with sodium hydroxide before flux measurement. X3257B2 fibers activated with FMP groups and capped with glycine had a hydraulic permeability of 0.7 $\text{Lm}^{-2}\text{hr}^{-1}\text{mmHg}^{-1}$. Treatment with NaOH gave a hydraulic permeability of 1.1 $\text{Lm}^{-2}\text{hr}^{-1}\text{mmHg}^{-1}$. XC3089B1 fibers functionalized with hydrazide groups had a hydraulic permeability of 0.35 $\text{Lm}^{-2}\text{hr}^{-1}\text{mmHg}^{-1}$. X3257B2 fibers activated with hydrazide groups had a hydraulic permeability of 5.5 $\text{Lm}^{-2}\text{hr}^{-1}\text{mmHg}^{-1}$.

Example 3: Attachment of Ligands to Functionalized Hollow Fibers**(a) Attachment of monoclonal or polyclonal antibodies to hydrazide-functionalized fibers**

5 Hydrazide-functionalized fibers obtained from Example 1(a) are reactive with oxidized carbohydrates. They were used for site-directed immobilization of antibodies containing carbohydrates primarily on the Fc portion of the molecule. The antibodies immobilized were as follows: (1) mouse monoclonal IgG1 antibody to human CD19 cell surface marker (Immunogen, 10 Inc., Cambridge, MA); (2) goat polyclonal IgG anti-mouse IgG (H and L) (Rockland, Inc., Gilbertsville, PA); and (3) mouse polyclonal IgG1 (Lampire Biological Laboratories, Pipersville, PA). The antibodies were oxidized under acidic pH conditions with sodium metaperiodate. The oxidized antibody was incubated with the fibers and the covalent bond was reduced and stabilized with sodium cyanoborohydride. The procedures followed are described in Holton, 15 O.D., Vicalvi, Jr., J.J., Optimization of Monoclonal Antibody Immobilization on Hydrazide-Preactivated Hollow Fiber Membrane, BioTechniques, 11:662-665 (1991). Fibers with immobilized ligand were stored at 4°C in PBS with 0.02% w/v sodium azide (PBSA) and 1% w/v bound serum albumin.

(b) Attachment of recombinant protein A to FMP-functionalized fibers

20 FMP-functionalized fibers obtained from Example 1(b) are reactive with amino groups. The covalent coupling was obtained by incubating recombinant protein A (rpA) (Repligen Corp., Cambridge, MA) with the fiber, using procedures described by Ngo, T.T., Facile Activation of Sepharose Hydroxyl Groups by 2-Fluoro-1 Methylpyridinium Toluene-4-Sulfonate: Preparation of Affinity and Covalent Chromatographic Matrices, BIO/TECHNOLOGY, 4:134-137 (1986), 25 followed by treatment with sodium hydroxide. Fibers with immobilized ligand were stored at 4°C in PBSA.

(c) Amount of ligand immobilized on the functionalized fibers

The amount of protein immobilized on the functionalized fibers was measured by incubating the protein-coated fibers with BCA protein assay reagent (Pierce, Rockford, IL), 30 measuring absorbance of the soluble colored product at 562 nm, and comparing measured values to a standard curve prepared with solutions containing known amounts of a protein the same as, or similar to, the immobilized protein.

(i) Attachment of antibodies to hydrazide-functionalized fibers

To measure antibody immobilized to hydrazide-functionalized fibers, controls, consisting

of the functionalized fiber with no immobilized protein, were included because components of the coating on the fiber reacted with the BGA protein assay reagent. The controls produced a baseline absorbance which was subtracted from the absorbance measured with immobilized protein. Monoclonal antibody (mAb) to CD19 was immobilized to hydrazide-functionalized fibers from both lots. As shown in Table 1, ligand densities ranged from 1×10^{10} - 3.2×10^{11} mAb/cm², where about 1×10^{12} mAb/cm² is a monolayer, assuming that each antibody occupies a square 100Å on each side. Table 1 also shows the fraction of the antibody initially present in the solution which was immobilized and the mean distance between ligand molecules on the fiber surface, assuming that the molecules were arranged in a square lattice. Non-immunospecific mouse IgG1 was immobilized to both lots of fibers to serve as controls for cell binding studies; the immobilization data was essentially the same as that shown for the mAb in Table 1. In addition, X3257B2 hydrazide-functionalized fibers with immobilized polyclonal goat anti-mouse IgG (G anti-M) (Rockland, Gilbertsville, PA) were prepared with a ligand density of 3.0×10^{11} Ab/cm² (see Table 1).

Table 1: Immobilization of Ligand

Protein	Immobilized (mg/mlmv)	Fraction Immobilized (%)	Ligand Density $\times 10^{-11}$ (molecules/cm ²)	Mean Distance Between Molecules (Å)
mAb anti-CD19	0.2 - 4.0	5 - 30	0.1 - 3.2	180 - 1000
G anti-M	3.1	34	3.0	192
rpA	7.8 - 9.1	43 - 56	27 - 35	53 - 61

(ii) Attachment of rpA to FMP-activated XC3257B2 Fibers

A BCA total protein assay was performed, as described above, to determine the amount of immobilized rpA. FMP-functionalized fibers were not reactive with the BCA assay so that protein amounts determined for these fibers were more accurate than for the hydrazide-functionalized fibers. rpA ligand densities ranged from $27-35 \times 10^{11}$ rpA/cm² (see Table 1), representing about a monolayer of protein, assuming that each rpA molecule occupies a square 50Å on each side.

(d) Binding capacity of immobilized ligand

(i) Determination of the binding capacity of X3257B2 FMP-functionalized, rpA-coated fibers for various proteins

Because the intended function of the spA-coated fibers was to bind cells that have been

coated with antibodies, the capacity of the fibers for specific adsorption of antibody and non-specific adsorption of inert proteins was tested. One cm sections of rpA-coated fiber was incubated with 1 ml of the target protein for several hours in a small test tube at room temperature. The fiber was then extensively washed with protein-free buffer until the wash buffer was determined to be free of protein as determined by UV absorption at 280 nm. The bound protein was then eluted with 50 mM glycine buffer in 150 mM NaCl at pH 2.5. Eluted protein was again quantified by absorption of UV light at 280 nm. Target proteins included human polyclonal IgG (Gammagard, Hyland Div., Baxter Healthcare, Glendale, CA), a mouse myeloma monoclonal IgG 2b (Sigma Chemical, St. Louis, MO), protein of the same isotype as the monoclonal antibody subsequently used in cell binding experiments, and bovine serum albumin (BSA) as a non-specific binding control. The results in Table 2, are expressed as the mean plus or minus one standard deviation, where n represents the number of measurements. The fibers had low non-specific adsorption, as indicated by their low capacity for BSA, but had high capacity for binding human IgG.

Table 2: Capacity Measurements For spA-coated X3257B2 FMP-functionalized Fibers

Protein	Capacity (mg/mlmv)
Human IgG	23 \pm 7 (n=6)
Mouse IgG2b	13 \pm 2 (n=4)
BSA	0.3 \pm 0.2 (n=3)

(ii) Determination of the binding capacity of X3257B2 hydrazide-functionalized, G anti-M-coated fibers for various proteins

Binding capacities of hydrazide-functionalized X3257B2 fibers coated with goat anti-mouse IgG were measured for various proteins in the same manner as described in Example 3(d)(i) in order to compare the specific binding of mouse IgG with non-specific binding of other proteins. Results shown in Table 3 indicate that these fibers showed significant non-specific binding of human IgG and BSA and that the capacity of these fibers for mouse immunoglobins was not distinguishable from non-specific binding with this assay.

Table 3: Capacity Measurements for G anti-M-coated X3257B2 Hydrazide-functionalized Fibers

5	Protein	Capacity (mg/mlmv)
	Human IgG	2.9
	Mouse IgG	7.3
10	Mouse IgG2b	3.1
	BSA	7.2

Example 4: Growth of Cell Lines

The two human cell lines used, Namalva (Ambrus, J.L. and Fauci, A.S., Human B lymphoma cell line producing B cell growth factor, J. Chem. Invest. 75:732-739 (1985), and HL-60 (Collins, S.J., Gallo, R.C., Gallagher, R.E., Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture, Nature 270:347-349 (1977)), were obtained from Dr. James D. Griffin, Dana Farber Cancer Institute, Boston, MA. The Namalva cell line is a B cell lymphoma cell line which expresses CD19 on its membrane but not CD33. The HL-60 cell line is a promyelocyte leukemia cell line which expresses CD33 but not CD19. The cell markers CD19 and CD33 were employed as targets for cell immobilization. The cell lines were grown in continuous culture at 37°C in a humidified air atmosphere containing 5% CO₂ in RPMI 1640 media (Moore, G.E., Gerner, R.E., Franklin, H.A., Culture of normal human leukocytes, JAMA 199:519-524 (1967)) supplemented with 25 mM HEPES buffer, 10% fetal bovine serum, 4 mM L-glutamine, 5000 IU/ml penicillin and 5000 µg/ml streptomycin. Cells were collected for experiments during their log growth phase.

Example 5: Direct Binding of Cells to Single Hydrazide- Functionalized XC3089B1 Hollow Fibers Coated with Immobilized Anti-CD19 mAb

Single hollow fibers were immobilized with anti-CD19 mAb to assess the feasibility of specifically binding and eluting Namalva target cells. Control experiments to assess non-specific binding included incubating the target cells (Namalva) with equivalent fibers, except that the fibers were coated with a non-immunospecific mouse IgG1 protein instead of anti-CD19, or incubating HL-60 cells (CD19-) with the fibers functionalized with anti-CD19 mAbs. Four or five cm lengths of hollow fibers containing immobilized ligand were prepared as described in

Examples 1 and 3. Physical properties were the same as described in Example 2. Ligand densities ranged from 1 to 3.2×10^{11} molecules/cm². FIG. 1 shows a schematic diagram of the functionalized hollow fibers that were used. Small sections of plastic (Tygon) tubing were glued to each end to facilitate pressurization. The plastic tubing was marked with a magic marker to allow the orientation of the fiber to be kept constant during observation. The cells from the culture (grown as described in Example 4) were stained with trypan blue dye, and live and dead cells were counted. The viability of the cultured cells prior to the start of the binding experiment averaged about 97% (range of 95% to 100%). The cells were washed twice in phosphate buffered saline pH 7.4 (PBS) at room temperature. The cells were resuspended in 5 ml PBS with 1% w/v BSA at a concentration of 5 million cells per ml and incubated at room temperature with the fibers in a T25 tissue culture flask having a growth area of 25 cm² which was tilted at a 45° angle in order to funnel cells onto the fiber surface. The fibers that were used had previously been washed extensively with phosphate buffered saline pH 7.4 (PBS) to remove any trace of sodium azide in the storage buffer. The cells settled onto the fiber by gravity during a one hour incubation at room temperature. The fluid was very gently disturbed by moving the flask back and forth at 15 minute intervals to resuspend unbound cells. The supernatant cell suspension was then gently aspirated, leaving enough residual liquid behind to completely cover the fiber. Fibers were then gently washed with 25 ml PBS very slowly (approximately 25 ml/min from a pipette), and the wash fluid was aspirated as described above. Washing was repeated three times. The fibers were then gently removed from the flask with tweezers by gripping the plastic tubing attached to the ends and quickly placed into a 60 mm or 100 mm diameter petri dish filled with PBS. Care was taken to perform transfers quickly and gently to minimize disruption of cells on the fiber by contact with air. The fibers were then observed with an inverted microscope in brightfield at low magnification (100x). Cells bound to the edge of the fiber were counted using a linear scale reticle. In those cases where the non-specific cell binding control experiment demonstrated a significant number of cells bound as compared to the test fiber, both fibers were flushed with a 3.5 ml transfer pipet in an identical fashion by aspirating some of the PBS in the dish and gently (about 1 ml/s flow) dispensing it along the length of the fiber so that the fluid stream was directly incident on the fiber edges being counted. The cells along the edge of the fiber were then recounted.

The cells were then removed by permeating buffered saline (pH 7.4) through the fiber for 2-5 minutes with a pressure of about 20 psig applied to the fiber lumen. Cell detachment

controls consisted of exposing one of the test fibers to identical handling conditions without pressurization (P=0 control). The procedure for pressurization was as follows. First, the fiber was carefully transferred from the petri dish in which the fiber had been observed under the microscope to a larger diameter dish filled with PBS. FIG. 2 shows the pressurization setup that was used. One end of the plastic tubing was sealed with a small polyethylene plug. To the other end was attached a small polyethylene connector that was in turn attached to plastic tubing connected to a 60 ml syringe in a syringe pump. Pressure in the tubing from the syringe was measured by a stainless steel pressure gauge connected via a T branch. With the tubing clamp closed, the syringe pump was turned on, thereby increasing the pressure in the line. When the pressure reached 20 psig, the clamp was released so that the fiber experienced the full pressure immediately. The pressure was maintained for 2-5 minutes at 20 psig by adjusting the syringe pump as necessary to keep a relatively constant pressure. During pressurization, the fiber was observed to determine if a gross leak was present, in which case the fiber was discarded. After pressurization, the fiber was detached from the pump tubing and transferred back to the petri dish used for observation, and the cells along the edges of the fibers were recounted with the fiber in the same orientation as when previously counted. Results for the XC3089B1 hydrazide-functionalized antibody coated fibers are shown in Table 4. The results from both sets of control experiments were similar, and the two sets are lumped together.

Table 4: Direct Binding and Removal of Cells From Hydrazide-Activated XC3089B1 Permeability Hollow Fibers with Immobilized Antibodies

<u>Binding</u>		<u>Removal</u>	
Cells bound/cm of fiber edge (mean \pm SEM)		Fraction removed of cells initially bound to test fibers (%) (mean \pm SEM)	
<u>Test</u>	<u>Control</u>	<u>P=20 psig</u>	<u>P=0 psig</u>
227 \pm 31	10 \pm 3	47 \pm 10%	11 \pm 3%
(n = 19)	(n = 20)	(n = 4)	(n = 3)

P = pressure

Specific cell attachment was successfully demonstrated in nineteen experiments with CD19+ cells binding to anti-CD19-derivatized fibers with an average of 23 times more cells bound than in control experiments. This result is equivalent to a selectivity for target cells of

95.6%. On average, less than half of the target cells were removed by pressurizing the fibers. The fibers lost their ability to specifically bind a significant number of cells after 2-3 uses.

Example 6: Direct Binding of Cells to Single Hydrazide-Functionalized XC3089B1 Hollow Fibers Coated with Immobilized Anti-CD19 mAb at Variable Ligand Density

Experiments equivalent to those described in Example 5 were performed using XC3089B1 hydrazide-functionalized fibers to which was immobilized protein mixtures containing anti-CD19 mAb and non-immunospecific mouse IgG1 in various ratios. In this way, total protein coverage on the fibers was maintained relatively constant while varying the surface concentration of specific ligand (anti-CD19). Results are shown in Table 5.

Table 5: Binding and Removal of Cells From XC3089B1 Hollow Fibers: Direct Coupling with Immobilized Monoclonal Antibodies at Variable Surface Concentration of Specific Antibodies

Specific Ligand Density	mAb/cm ²	Binding Cells bound/cm of fiber edge (mean \pm SEM)	Removal	
			Fraction removed of cells initially bound to test fibers (%) (mean \pm SEM)	
			P = 20 psig	P = 0 psig
20	1 x 10 ¹¹	250 \pm 50 (n = 6)	50 \pm 10 (n = 4)	11 \pm 3 (n = 3)
	1.3 x 10 ¹⁰	370 \pm 60 (n = 7)	60 \pm 15 (n = 4)	10.7 \pm 0.3 (n = 2)
25	7.8 x 10 ⁹	200 \pm 50 (n = 7)	7 and 80 (n = 2)	80 \pm 15 (n = 2)
	0 (control)	14 \pm 7 (n = 7)		

Fibers with 1.3 x 10¹⁰ mAb/cm² behaved in a similar fashion to fibers coated with undiluted anti-CD19 at 1 x 10¹¹ mAb/cm². With a further reduction to 7.8 x 10⁹ mAb/cm², one fiber yielded 80% removal and the other yielded 7% removal. Thus, at the lowest ligand density, cells removed by pressurization were no longer substantially greater than those removed by simple handling.

Example 7: Direct Binding of Cells to Single Hydrazide-Functionalized X3257B2 Hollow Fibers Coated With Immobilized Anti-CD19 mAb

Experiments were conducted with the X3257B2 fibers having ligand densities ranging from 0.8 to 3.2×10^{11} mAb/cm² which is similar to ligand densities obtained with the XC3089B1 fibers. The protocol was identical to that described in Example 5 with the following changes. After the fiber was attached to the pump for pressurization and before releasing the clamp, the fibers (attached to the pump tubing) were quickly transferred to polypropylene centrifuge tubes filled with sufficient buffer or culture media (RPMI 1640 containing 1% w/v BSA or 10% v/v fetal bovine serum) to completely submerge the fibers (FIG. 3). This change facilitated collection of the detached cells which were subsequently concentrated by centrifugation and assayed for viability by trypan blue dye exclusion. Instead of including two test fibers, one of which is pressurized at 20 psig and the other at 0 psig as a handling control, a single test fiber was used as follows. After incubation with the cells, both the single test fiber and the non-specific binding control fiber were observed, and cells were counted along the edges. The single test fiber was then attached to the pump tubing and transferred to the centrifuge tube filled with cell-collection buffer but not pressurized. The fiber was then observed again in the same orientation and cells were recounted. The number of cells present was compared to the amount counted in the previous step and the percent of cells removed is indicated as the P=0 control. The same test fiber was then attached to the pump tubing and pressurized at 20 psig. The fiber was observed again, and the cells were counted. The percent of cells removed in this step is indicated as the amount removed by pressurization. Cells removed from the fiber were collected for some experiments and stained with 0.2% trypan blue in PBS to determine recovered cell viability. The results of these experiments are shown in Table 6.

Table 6: Binding and Removal of Cells From Hydrazide-Activated X3257B2 Hollow Fibers: Direct Coupling with Immobilized Monoclonal Antibodies

Binding	Removal		Viability
	Fraction removed of cells initially bound to test fibers (%) (mean \pm SEM)		Fraction viable of recovered, eluted cells (%) (mean \pm SEM)
Test Control	P = 20 psig	P = 0 psig	P = 20 psig
500 \pm 120 (n = 5)	86 \pm 5% (n = 5)	32 \pm 12% (n = 5)	90 \pm 10% (n = 2)
33 \pm 18 (n = 5)			

Specific adsorption of target cells, as well as non-specific adsorption of non-target cells was higher with X3257B2 fibers than for the XC3089B1 fibers with a ratio of specific binding to non-specific binding of about 15 to 1. This is equivalent to a target cell selectivity of 93.4%. The fraction of target cells removed by pressurization was substantially higher with X3257B2
5 fibers than with XC3089B1 fibers. Because the former fibers had a 15-fold higher hydraulic permeability than the latter, these data indicate that hydraulic permeability is an important characteristic for fiber performance in cell separation.

In one experiment, the cells were stained with a fluorescent vital stain (10 μ M Cell Tracker Orange, Molecular Probes, Eugene, OR) prior to incubation with the fibers. In addition
10 to counting the cells along the fiber edge in bright field, the cells on the fiber were also visualized *en face* with epi-fluorescence. Images were captured with a CCD video camera and were analyzed on an Apple Macintosh Qudra 950 using NIH Image software. Images were captured from five specifically defined areas of the fiber after incubation and washing and after vigorous flushing of the fiber with a pipette to remove cells by shearing. Cell densities
15 determined from the captured images before and after shearing were compared in order to quantify the percent of cells removed by shearing. Image analysis indicated that 47% of the cells were removed compared to 55% removed as determined by counting cells along the fiber edge. Thus, the two methods of quantifying cell adsorption are in reasonable agreement.

20 **Example 8: Indirect Binding of mAb-coated Cells to Single FMP-functionalized X3257B2 Hollow Fibers Coated with Immobilized rpA**

Experiments were performed using FMP-functionalized X3257B2 rpA-coated fibers which utilized an indirect or "sandwich" method of cell immobilization. The objective was to
25 demonstrate selective binding of HL-60 target cells coated with anti-CD33 mAbs and to determine if cells can be removed by pressurization with neutral and low pH buffers with these fibers. The protocol for these experiments was similar to that in Example 7 except that the cells were first incubated with mAb before contact with the fibers. Specific binding was studied with CD33+ HL60 target cells which were incubated with anti-CD33 mAb and then brought into
30 contact with the fibers. Non-specific binding control experiments included incubation of the fibers with Namalva cells (CD33-) which had first been pre-incubated with anti-CD33 and incubation of the fibers with HL-60 cells which had first been pre-incubated with a non-immunospecific mouse antibody of the same isotype as the anti-CD33 (IgG2b). Results from

both control experiments were similar and were lumped together. The cells in culture were counted, and viability was determined with trypan blue exclusion. The cells were washed with cold (4°C) RPMI media containing 10% FBS. The cells were then resuspended at a concentration of 10^7 cells/ml in the same media but containing 10 µg/ml mAb. The cells were incubated in the cold in this solution for 30 min and then washed twice with cold PBS and resuspended at 5×10^6 cells/ml in cold PBS with 1% w/v BSA. This cell suspension was then incubated with the fiber in the cold for 30 min and then at room temperature for an additional 45 min. The remainder of the protocol was the same as that described in Example 7, except that for some experiments, fibers were pressurized with 50 mM glycine buffer containing 150 mM NaCl, pH 2.5, instead of PBS at pH 7.4. Removed cells were collected in centrifuge tubes as described in Example 7 containing a large enough volume of media supplemented with 25 mM HEPES buffer to maintain final pH high enough (pH \geq 6.6) so as to minimize loss of viability of the collected cells. The results of these experiments are shown in Table 7.

Table 7: Binding and Removal of Antibody-coated Cells From X3257B2 Hollow Fibers: Indirect Coupling With Immobilized Protein A

20	Binding		Removal			Viability		
	Cells bound/cm of fiber edge (mean \pm SEM)		Fraction removed of cells initially bound to test fibers (%) (mean \pm SEM)			Fraction viable of recovered, eluted cells (%) (mean \pm SEM)		
	Test	Control	P = 20 psig (pH 7.4)	P = 20 psig (pH 2.5)	P = 0 psig	P = 20 psig (pH 7.4)	P = 20 psig (pH 2.5)	P = 0 psig
25	388 \pm 7 (n=6)	0.6 \pm 0.4 (n=3)	73 \pm 10 (n=3)	96 \pm 2 (n=3)	56 \pm 11 (n=6)	80 \pm 10 (n=3)	82 \pm 5 (n=3)	97 \pm 2 (n=4)

The cells bound specifically at levels comparable to experiments which employed direct binding. Non-specific adsorption was lower with these fibers than observed with the hydrazide-functionalized fibers. Over 600 times more target cells bound to fibers than did non-target cells. This result is equivalent to a selectivity of 99.8%. The percent removal of target cells by pressurization was midway between the performance of the XC3089B1 and X3257B2 hydrazide fibers. The hydraulic permeability of the FMP-functionalized fibers was about three times that of the XC3089B1 hydrazide fibers, but was only about one-fifth that of the X3257B2 hydrazide

fiber. The fact that the performance was close to that of the higher flux X3257B2 hydrazide fibers at neutral pH (Table 6) along with the observation that a large percentage of cells were removed by handling (P=0 control), suggests that the target cells were more loosely attached with X3257B2 hollow fibers containing immobilized rpA. Elution with a low pH buffer removed practically all of the attached cells.

Example 9: Purification of HL-60 Target Cells from a 50:50 Mixture of HL-60 and Namalva Cells Using Indirect Binding to Single FMP-functionalized X3257B2 Hollow Fibers with Immobilized rpA

Experiments were performed using FMP-activated X3257B2 fibers coated with rpA and mixed cell populations to directly observe the selectivity of the fibers for target cells. HL-60 and Namalva cells in culture were counted, centrifuged at 420xg to pellet cells, and then resuspended in RPMI 1640 media containing one of two fluorescent vital stains (10 μ M Cell Tracker Orange or 6 μ M Cell Tracker Green, Molecular Probes). Each cell line was incubated with a different stain so that they would fluoresce in different colors. The stained cells were then washed in cold RPMI 1640 with 10% v/v FBS and incubated with anti-CD33 mAb as described in Example 8. Immediately prior to incubation, the two cell lines were mixed together to yield a 50:50 mixture. This mixture was then incubated with the fiber as described in Example 8. After incubation with the fiber and gentle washing, the fiber was observed under epi-fluorescence and several areas with bound cells were photographed. Each area was first photographed with a filter set appropriate for one stain and then with another filter set appropriate for the other stain. Cells observed on the fiber were subsequently counted by projecting the processed color slides onto a screen and manually counting the cells of each type. The fiber was then pressurized and cells recovered in the centrifuge tube were centrifuged and resuspended in a small volume. A small aliquot was placed on a glass microscope slide, covered with a cover slip, and the cells were observed in the same way as the cells on the fiber, and the number of each type was determined by similar manual counting. The purity of the target cells (HL-60) in each case was determined from these cell counts. Purity of the target cells bound to the fibers was $99.1 \pm 0.4\%$ (mean \pm SEM for n=3). Purity of the recovered eluted cells was $90.3 \pm 1.6\%$ (n=4).

Example 10: Indirect Binding of mAb-coated Cells to Single Hydrazide-functionalized X3257B2 Hollow Fibers Coated with Goat Anti-Mouse IgG

Binding of HL-60 target cells pre-incubated with anti-CD33 monoclonal antibody to
5 hydrazide-activated X3257B2 fibers coated with Goat anti-Mouse IgG was determined. The
procedure followed was the same as that described in Example 8, except that the bound cells
from the fibers were not removed. The results were that 299 target cells (HL-60)/cm fiber length
were attached to the test fiber. The level of binding for the non-specific binding control
(Namalva cells) was 44 cells/cm fiber length. Thus, about 6.8 times more target cells bound than
10 non-target cells. This result corresponds to selectivity for target cells of 85.3%. These results
indicate that the interaction between the Goat anti-Mouse IgG and the target protein was stronger
than the interaction with a non-specific protein.

Example 11: Visualization of Cell Attachment

15 The morphology of vital and aldehyde-fixed, specifically and non-specifically adhered
cells on fibers was observed using an Environmental SEM. This method gives a high level of
magnification and detail. Images obtained indicated that there was a significant flattened region
of the cell in intimate contact with the fiber.

20 Those skilled in the art will be able to ascertain, using no more than routine
experimentation, many equivalents of the specific embodiments of the invention described
herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for separating a mixture of cells in suspension having a first cell type and a second cell type, comprising:

providing a suspension of cells, said suspension having a first cell type and a second cell

5 type;

providing a porous material having a bound ligand, said bound ligand being able to additionally bind to said first cell type;

contacting said porous material having said bound ligand with said suspension of cells under conditions which allow binding of said first cell type to said bound ligand so as to form a

10 porous material-ligand-cell complex;

treating said porous material-ligand-cell complex so as to substantially remove said second cell type from said porous material;

applying a back pressure across said porous material-ligand-cell complex so as to detach said first cell type from said porous material-ligand-cell complex; and

15 recovering said detached first cell type.

2. The method of claim 1 wherein said porous material is a membrane.

3. The method of claim 1 wherein said porous material has surface pores of a size so as to preclude passage of said cells therethrough.

20

4. The method of claim 1 wherein said condition in said contacting step comprises applying a forward pressure across said porous material so as to induce normal filtration.

5. The method of claim 1 wherein treating said porous material-ligand-cell complex comprises washing said complex.

25

6. The method of claim 1 wherein a substantial portion of said detached first cell type are viable.

7. The method of claim 1 further comprising applying an additional parameter so as to aid in said detachment of said first cell type, said additional parameter being selected from the group consisting of a shear force, an electrical force, a centrifugal force, a chemical, a biological molecule, a pH change, an ionic strength change and mixtures thereof.

5

8. A method for detaching cells that are adsorbed to a porous material, comprising:
providing a porous material having adsorbed cells;
applying a first back pressure across said porous material so as to detach said adsorbed cells from said porous material; and
10 recovering said detached cells.

9. The method of claim 8 wherein said porous material has a bound ligand and wherein said cells are adsorbed to said porous material as a result of said cells being bound to said ligand.

15 10. The method of claim 8 wherein said adsorbed cells comprise a first cell type and a second cell type, said first cell type being adsorbed to said porous material with a higher affinity than said second cell type, and wherein a second back pressure across said porous material is applied after application of said first back pressure such that said first back pressure selectively detaches said second cell type and said first second back pressure selectively detaches said first cell type.

20

1/2

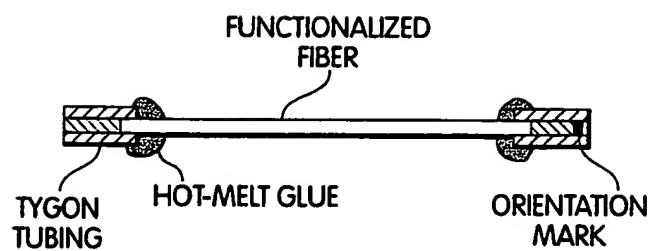


Fig. 1

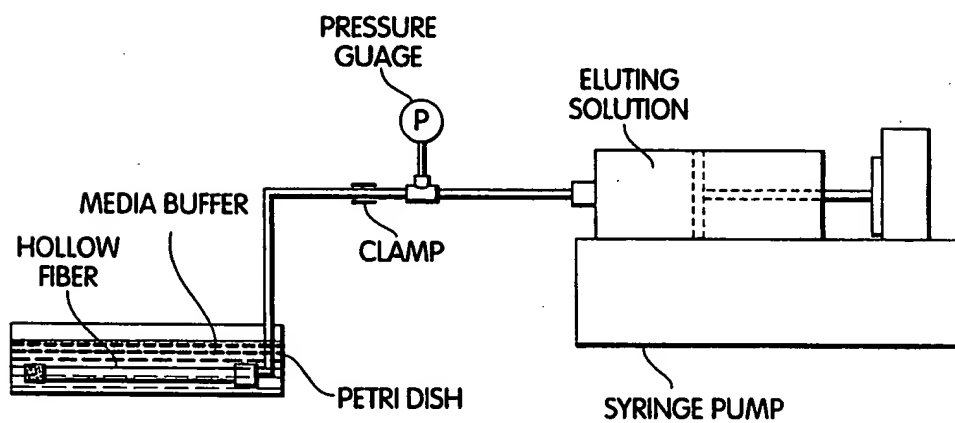


Fig. 2

2/2

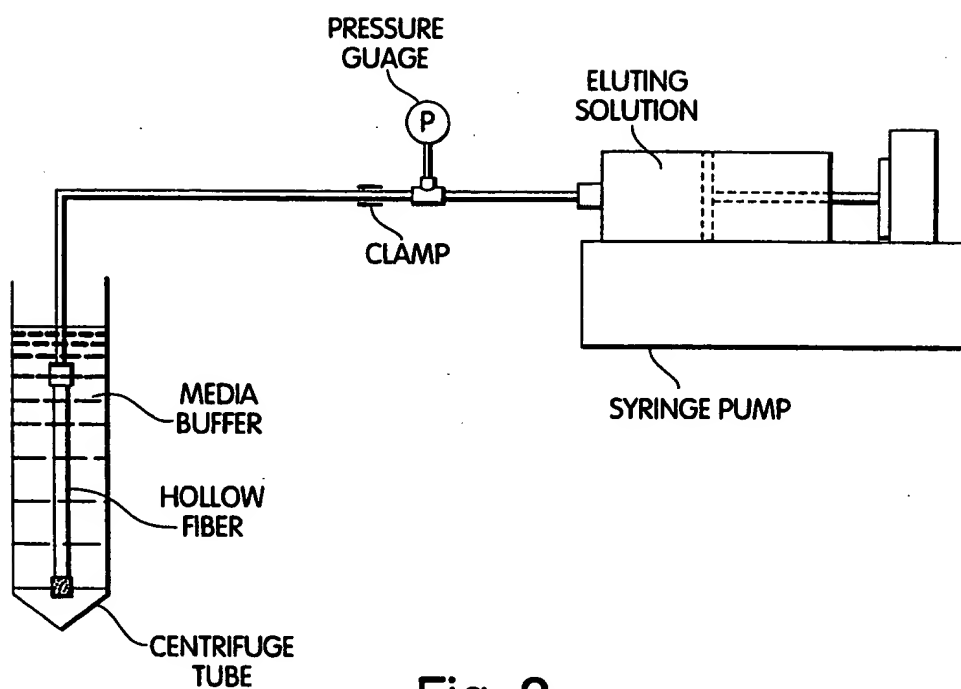


Fig. 3

INTERNATIONAL SEARCH REPORT

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 PCT/US96/13361
A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 1/18, 33/543, 33/544, 33/537

US CL : 536/518, 530, 538, 177, 178, 824

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/518, 530, 538, 177, 178, 824

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, WPIDS

search terms: cell separation, pressure, force, eluting, affinity, filter, viable

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,310,688 A (ZALE ET AL) 10 May 1994 (10.05.94), see entire document.	1-10
X	US 5,003,047 (YARMUSH ET AL) 26 March 1991 (26.03.91), see entire document.	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z

document member of the same patent family

Date of the actual completion of the international search

01 OCTOBER 1996

Date of mailing of the international search report

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